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An Orc1/Cdc6 ortholog functions as a key regulator in the DNA damage response in Archaea

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ABSTRACT

While bacteria and eukaryotes show distinct mechanisms of DNA damage response (DDR) regulation, investigation of ultraviolet (UV)-responsive expression in a few archaea did not yield any conclusive evidence for an archaeal DDR regulatory network. Nevertheless, expression of Orc1-2, an ortholog of the archaeal origin recognition complex 1/cell division control protein 6 (Orc1/Cdc6) superfamily proteins was strongly activated in *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius* upon UV irradiation. Here, a series of experiments were conducted to investigate the possible functions of Orc1-2 in DNA damage repair in *Sulfolobus islandicus*. Study of DDR in Δ *orc1-2* revealed that Orc1-2 deficiency abolishes DNA damage-induced differential expression of a large number of genes and the mutant showed hypersensitivity to DNA damage treatment. Reporter gene and DNase I footprinting assays demonstrated that Orc1-2 interacts with a conserved hexanucleotide motif present in several DDR gene promoters and regulates their expression. Manipulation of *orc1-2* expression by promoter substitution in this archaeon revealed that a high level of *orc1-2* expression is essential but not sufficient to trigger DDR. Together, these results have placed Orc1-2 in the heart of the archaeal DDR regulation, and the resulting Orc1-2-centered regulatory circuit represents the first DDR network identified in Archaea, the third domain of life.

INTRODUCTION

Organisms of all three domains of life have to deal with lesions on their chromosomal DNAs generated by environ-

mental and endogenous factors. If left unrepaired, these DNA lesions will either alter the content of the genetic blueprint, giving rise to mutations, or leading to the loss of genome integrity and cell death. DNA damage repair has been studied in great detail in the organisms belonging to the domains of Bacteria and Eukarya, in which distinctive regulatory networks called DNA damage response (DDR) have been revealed (1,2). The bacterial DDR is best represented by the SOS response, which is triggered by ssDNA-RecA filaments, an intermediate of homologous recombination repair (HRR) of double stranded DNA breaks (DSBs). Investigation of the SOS regulation in *Escherichia coli* has revealed a LexA-dependent regulatory network that is conserved in numerous bacteria (3). Nevertheless, several LexA-independent mechanisms have also been discovered in different bacteria, revealing the diversification of the bacterial DDR regulatory mechanisms (4). DDR regulation in eukaryotes is far more complex than those found in bacteria, involving multiple genome surveillance mechanisms. Two best-known examples are the ataxia telangiectasia-mutated (ATM) and the ATM and Rad3-related (ATR) signal transduction pathways that control, for example, cell cycle checkpoint regulation (5,6). Nevertheless, all these bacterial and eukaryotic DDR mechanisms share some common features; after recognition of DNA damage signal, a series of cellular events occur in a coordinated fashion, including inhibition of DNA replication, cell cycle arrest and activation of the synthesis of various DNA repair enzymes, and such a regulation ensures efficient DNA damage repair to occur in a timely fashion in the cell.

Investigation of UV-responsive genome expression in a few model archaea including *Halobacterium* NRC-1 (7,8), *Sulfolobus solfataricus* P2 (9–11) and *Sulfolobus acidocaldarius* DSM639 (10) has revealed that a number of genes are differentially expressed. Nevertheless, uncertainty about whether these observations reflect the presence of an ar-

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chaical DDR regulation persists primarily because of two reasons: (a) The differentially expressed genes (DEGs) exclude those coding for the enzymes responsible for nucleotide excision repair (NER), which are strongly activated by DNA damage in bacteria and eukaryotes (12,13), and (b) archaea do not code for any homologs of bacterial or eukaryotic DDR regulators. As a result, it remains as an open question whether organisms of the archaeal domain possess any DDR regulation, and if so, how the process is controlled.

In this work, we employed *S. islandicus*, a genetic model in the Crenarchaeota (14) to investigate the DNA damage-responsive genome expression and its regulation. Previous genetic analysis of three *orc1* genes in *S. islandicus* showed that *orc1-1* and *orc1-3* code for replication initiators responsible for replication initiation from the *oriC1* and *oriC2* of the chromosome but deletion of *orc1-2* gene does not impair the origin usage in this archaeon (15). The strong up regulation of *orc1-2* in *S. solfataricus* and *S. acidocaldarius* (upon UV irradiation (9,10)) and in *S. islandicus* (by treatment of 4-nitroquinoline 1-oxide (NQO) and UV light (16)) prompted us to investigate possible functions of *Orc1-2* in DNA damage response in this crenarchaeon. We found that *Orc1-2* has gained a novel function during evolution, i.e. the factor functions as a global regulator in the DNA damage response network of *S. islandicus*.

MATERIALS AND METHODS

Growth conditions, transformation and NQO treatment of *Sulfolobus*

S. islandicus strains used in this work are listed in Supplementary Table S1. *Sulfolobus* cells were cultured at 78°C in SCV (basic salts plus 0.2% sucrose, 0.2% casamino acids, 1% vitamin solution) medium (17) or in ACV medium (in which sucrose is replaced with D-arabinose). If required, uracil was added to 20 µg/ml. Plasmids were introduced into *S. islandicus* strains by electroporation as described originally for *S. solfataricus* (18).

NQO treatment experiments were conducted as previously described (16). A stock of the drug (130 mM) was prepared with DMSO and kept in -20°C. By the time of NQO experiment, the stock solution was diluted to 1.3 mM with H₂O. Then, the diluted NQO solution was added to *Sulfolobus* cultures of an early exponential growth phase (i.e. absorbance at 600 nm (A_{600}) = ca. 0.2) to the concentrations specified in each experiment. Cell samples were taken during incubation and used for A_{600} measurement, cell aggregation assay, cell viability assay, RNA extraction, western blot analysis and flow cytometry individually.

Cell viability of *Sulfolobus* cultures was estimated by determination of their colony formation units (CFU). Cells were collected from 1 ml of culture by centrifugation for each cell sample and re-suspended in the equal volume of pre-warmed SCV medium. Each cell resuspension was diluted in series of dilutions and plated for colony formation using the two-layer plating method previously described (19). Colonies of *Sulfolobus* cells appeared on plates after 7 days of incubation.

Transcriptome analysis by RNA sequencing and verification of the data by RT-qPCR

Exponentially growing cultures of *S. islandicus* E233S1 (the wild-type strain, WT,) and $\Delta orc1-2$ were diluted to an A_{600} of ca. 0.2 and grown in the presence or absence of 2 µM NQO for 6 h. Cell mass was then collected by centrifugation and employed for extraction of total RNAs using the Trizol reagent (Ambion, Austin, TX, USA). The quality and quantity of the total RNA preparations were evaluated using NanoDrop 1000 spectrophotometer (Labtech, Wilmington, MA, USA) and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA sequencing (RNA-Seq) was conducted in Novogene (Beijing, China). About 3 µg of high quality RNA were used for construction of RNA-Seq libraries, which were then subjected to next generation sequencing using an Illumina HiSeq2000. This gave 4.1–4.8 million high quality sequence reads for each RNA sample, which were then mapped to the genome of *S. islandicus* Rey15A (20). The resulting data were then analyzed by Fragments Per Kilobase of transcript sequence per Million base pairs sequenced (FPKM) analysis (21) to reveal expression levels of all genes in the *S. islandicus* genome. Differential genome expression analysis (NQO-treated samples versus the corresponding untreated references) was performed using the DEGSeq R package (22). Corrected P-value of 0.005 and log₂ (Fold change) of 1 were set as the threshold for significant difference in differential gene expression.

Quantitative reverse transcription PCR (RT-qPCR) was employed to validate the RNA-Seq data. DDR genes chosen for verification included *dpo2*, *upsX*, *upsA*, *tfb3*, *SiRe_1957* and *SiRe_1550*. First-strand cDNAs were synthesized with total RNA samples using a reverse transcriptase (Thermo-Scientific, Waltham, MA, USA) and random hexamer primers. The resulting cDNA samples were used for estimation of mRNA levels of the above DDR genes by qPCR, using the Maxima SYBR Green/ ROX qPCR Master Mix (Thermo Scientific, Waltham, MA, USA) and gene-specific primers (Supplementary Table S2). PCR was performed in a CFX96 Touch™ real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with the following steps: denaturing at 95°C for 5 min, 40 cycles of 95°C 15 s, 55°C 15 s and 72°C 20 s. Relative amounts of mRNAs were estimated by using the comparative Ct method with 16S rRNA as the reference (23). A correlation between the two sets of data was found to be 0.9705 with an *R*-value of 0.96 (Supplementary Figure S1).

Cell aggregation assay and flow cytometry

The extent of cell aggregation in *S. islandicus* cultures was estimated by direct observation of cell aggregates in fresh cultures under a Nikon Eclipse Ti-E inverted microscope (Nikon, Kobe, Japan). Data were collected from at least 12 fields of view images and 500 single cells for each cell sample, and the same analysis was conducted for three independent growth experiments.

Flow cytometry of *S. islandicus* cells was conducted as previously described (24). Briefly, the archaeal cells were fixed with ice-cold ethanol, stained with 40 µg/ml ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA) and 100

μg/ml mithramycin A (Apollo Chemical, Tamworth, UK) and analyzed in an Apogee A40 cytometer (ApogeeFlow, Hertfordshire, UK) equipped with a 405 nm laser. A dataset of at least 60 000 cells was collected for each cell sample.

Construction of *S. islandicus* *orc1-2*^{araS} mutant using a CRISPR-based genome-editing method

The genome-editing plasmid pGE-*orc1-2*^{araS} was constructed by following the strategy previously described (25). A target site was selected on the *orc1-2* gene, consisting of a 5'-CCN-3' (positioned at -28 referring to the start codon of *orc1-2*), a type I-A protospacer adjacent motif and the immediately downstream 40-nt DNA sequence (protospacer). Two DNA oligonucleotides were then designed based on the protospacer, giving *orc1-2*^{araS} Spacer F and *orc1-2*^{araS} Spacer R (Supplementary Table S2). Annealing of the two oligonucleotides yielded a DNA fragment containing the designed spacer. The resulting DNA fragment also contained the 5'-flanking 4 nt protruding ends that are compatible to the ends of the *SapI*-digested pSe-Rp vector. Therefore, ligation of the spacer fragment and the digested vector yielded the mini-CRISPR plasmid pAC-*orc1-2*.

The donor DNA fragment was generated by splicing and overlapping extension (SOE)-PCR with Fast Pfu DNA polymerase (TransGene, Beijing, China), following the published procedure (26). First, *orc1-2* gene was amplified by PCR from the genome DNA using the primer pair of *orc1-2* fwd-NcoI/*orc1-2* rev-XmaI. Insertion of the PCR fragment into pZC1-S-50-*orc1-2* yielded pSe-*araS-orc1-2* carrying the *araS-orc1-2* fusion gene. Then, a 654-bp genomic DNA fragment was obtained from the archaeal genome by PCR with the primer pair of *orc1-2*^{araS} SOE fwd-SphI/*orc1-2*^{araS} SOE rev whereas the fusion gene was amplified by PCR from pSe-*araS-orc1-2* with the primer pair of *orc1-2*^{araS} SOE fwd/*orc1-2*^{araS} rev-XhoI. Finally, the two PCR fragments were joined together by PCR using the primer pair of *orc1-2*^{araS} fwd-SphI/*orc1-2*^{araS} rev-XhoI. The resulting DNA fragment was digested with SphI and XhoI and inserted into pAC-*orc1-2* at the same sites, giving pGE-*orc1-2*^{araS}. The identity of the plasmid was confirmed by sequence determination of the DNA inserts by DNA sequencing.

pGE-*orc1-2*^{araS} was then introduced into *S. islandicus* E233S1 by electroporation, giving transformants that were subjected to *pyrEF* counter selection to cure the pGE plasmid as previously described (19). The genotype of single colonies obtained on 5-FOA-containing plates were determined by PCR amplification of the insert with *orc1-2*^{araS} check-fwd/*orc1-2*^{araS} check-rev primers and subsequent sequencing of the PCR products. One of the verified mutants was designated as *S. islandicus* *orc1-2*^{araS} and used in subsequent experiments.

Construction of reporter gene plasmids and report gene assay of DDR gene promoters

Reporter plasmids were constructed using the *Sulfolobus*-*E. coli* shuttle vector pSeSD (27) with the *S. solfataricus* β-glycosidase gene (*lacS*) (28) as the reporter gene. Four highly up-regulated genes (SiRe.1881: *upsA*, SiRe.1879:

upsE, SiRe.1717: *tfb3* and SiRe.1316: *cedA1*) were selected for the experiment. Promoter fragments of these genes were amplified by PCR with Fast Pfu DNA polymerase (TransGene, Beijing, China) from the genomic DNA of *S. islandicus* REY15A (20), using the following primer pairs individually, *upsA*-fwd-SphI/*upsA*-rev-NdeI, *upsE*-fwd-SphI/*upsE*-rev-NdeI, *tfb3*-fwd-SphI/*tfb3*-rev-NdeI or *cedA1*-fwd-SphI/*cedA1*-rev-NdeI (Supplementary Table S2). The resulting PCR products (ca. 220 bp) were purified with the GeneJET PCR Purification kit (Thermo Scientific, Waltham, MA, USA), and the purified DNAs were digested with *SphI* and *NdeI* and purified again. Each purified promoter fragment was then inserted into pSe-*lacS* (27), yielding pSe-*upsA*-*LacS*, pSe-*upsE*-*LacS*, pSe-*tfb3*-*LacS* and pSe-*cedA1*-*LacS* reporter gene plasmids containing the promoters of the wild-type DNA damage responsive element (DDRE) (Supplementary Table S1). Promoter derivatives containing the mutated DDRE (DDRE^{mut}) motifs were constructed using the SOE-PCR procedure (29). First, two overlapping primers were designed containing desired mutations in the center of each pair of oligonucleotides (Supplementary Table S2), including *upsA*-SOE-top/*upsA*-SOE-bm, *upsE*-SOE-top/*upsE*-SOE-bm, *tfb3*-SOE-top/*tfb3*-SOE-bm and *cedA1*-SOE-top/*cedA1*-SOE-bm. These SOE primers and their corresponding promoter-cloning primers were then employed for SOE-PCR with the corresponding reporter gene plasmid as template. The resulting DDRE^{mut} promoter fragments were then inserted into pSe-*lacS* (27), giving four reporter gene plasmids with DDRE^{mut} promoters, i.e. pSe-*upsA*^{mut}-*LacS*, pSe-*upsE*^{mut}-*LacS*, pSe-*tfb3*^{mut}-*LacS* and pSe-*cedA1*^{mut}-*LacS* (Supplementary Table S1). Determination of the sequences of the plasmid-borne wild-type and mutated promoters of the four DDR genes by DNA sequencing confirmed the identity of these reporter gene plasmids.

These reporter gene plasmids were then introduced into *S. islandicus* E233S1 (WT) and Δ*orc1-2* individually by electroporation. Three colonies of transformants were chosen from each transformation and grown for 6 h in SCV either in the presence or absence of 2 μM NQO. Cell mass was then collected from which cell extracts were prepared by sonication. β-Glycosidase activity in the cell extracts of different *S. islandicus* strains was determined using the ONPG (p-nitrophenyl-β-D-galactopyranoside) method as described previously (30).

Western blotting and hybridization analysis

Cells in 15 ml culture were collected by centrifugation. Cell pellets were re-suspended in 1 ml 10 mM Tris-HCl buffer (pH 8.0). The resulting cell suspensions were sonicated to disrupt *Sulfolobus* cells. Cell debris was removed by centrifugation 13000 rpm at 4°C for 30 min, yielding cellular extracts for further analysis. Protein concentrations of the samples were determined using Coomassie Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Similar amounts of protein (ca. 10 μg) were taken from the prepared cell extracts and loaded on a 12% polyacrylamide gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, fractionated proteins were transferred from the polyacrylamide gel onto a ni-

Table 1. Survival rates of *S. islandicus* wild-type strain (E233S1) and Δ orc1-2 after NQO treatment

Doses of NQO (μ mol/l)	Viable cells in % after NQO treatment ^a		Ratio
	E233S1	Δ orc1-2	
0	100	100	1.0
1	42.66	11.33	3.8
2	11.47	1.92	6.0
2.5	3.29	0.23	14.3
3	1.23	0.04	30.8
4	0.04	0.002	20.0

^aExponentially growing cultures were treated with different doses of NQO for 6 h. Cell samples were taken and plated on drug-free SCVU plates for determination of colony formation units (CFU). Survival rates are expressed as % of viable cells in drug-treated cultures relative to those in the corresponding drug-free reference cultures.

trocellulose blotting membrane (GE Healthcare, Wauke-sha, WI, USA), using the Semi-Dry Electrophoretic Trans-fer Cell system (Bio-Rad, Hercules, CA, USA) and used for immunoblotting. Briefly, the membrane was incubated in 5% skim milk blocking agent for 1 h, and then incu-bated with individual primary rabbit antibodies (against Orc1-2 or PCNA3 proteins) and finally with the horseradish peroxidase-labeled goat anti-rabbit antibody (Beyotime, Beijing, China) as described previously (31). Protein bands were visualized using the ECL western blot substrate (Thermo Scientific, Waltham, MA, USA) and recorded by exposure to an X-ray film.

Sequence analysis of promoters of highly activated DDR genes

Promoter sequences (100 bp preceding the start codon) of 21 Orc1-2-depended NQO-responsive genes (over 16 folds change after NQO treatment, Supplementary Table S6) were individually retrieved from the genome sequence of *S. islandicus* REY15A (20). These sequences were then used for *de novo* motif discovery by using MEME (Multiple EM for Motif Elicitation) with the default setting to identify conserved motifs as previously reported (32).

DNase I footprinting assay

Orc1-2 protein was expressed in *E. coli* Rosetta cells carry-ing pET-orc1-2. The *E. coli* strain was cultured in an LB medium containing 30 μ g/ml kanamycin at 37°C until A_{600} = 0.6. Orc1-2 protein synthesis was induced by adding 0.5 mM IPTG, and the induction was for 12 h at 16°C. Cell mass was harvested by centrifugation and re-suspended in the lysis buffer (50 mM phosphate saline buffer, 500 mM NaCl, 20 mM imidazole). Cells were disrupted using a French press at 4°C. After removing cell debris by centrifugi-tation, the supernatant was loaded onto a 1 ml Ni-NTA col-umn (GE Healthcare, Chicago, Illinois, USA). His-tagged recombinant Orc1-2 protein was purified by following the manufacturer’s instruction.

DNase I footprinting assay was performed as described previously (33). The *upsE* and *tfb3* original promoter P_{upsE}-DDRE and P_{tfb3}-DDRE and the mutated promoter P_{upsE}-DDRE^{mut} and P_{tfb3}-DDRE^{mut} were amplified individually

from the corresponding reporter gene plasmids (Supple-mentary Table S1) using primers listed in Supplementary Table S2. The resulting PCR products were then cloned into pCR™4-TOPO® (Thermo Scientific, Waltham, MA, USA), yielding plasmids that were used as template for PCR with an M13fwd primer carrying a 5’-end FAM la-beling and an M13rev primer carrying a HEX 5’-end label-ing. The resulting fluorescence-labeled DNAs were used as DNA probes for DNase I footprinting analysis. The coding strand of the *upsE* promoter region was labeled by FAM (peaks above the promoter sequences) and the non-coding strand was labeled by HEX (peaks below the promoter se-quence). The two DNA strands of the *tfb3* promoter re-gion were labeled in the opposite combination: HEX for the coding strand and FAM for the non-coding strand. To ensure efficient binding, 400 ng of labeled DNA and 13.7 μ g of Orc1-2 protein were mixed and incubated at 40°C for 20 min in 50 μ l binding buffer [20 mM HEPES (pH 7.6), 10 mM (NH₄)₂SO₄, 1 mM DTT, 0.2% Tween-20, 30 mM KCl]. Then, 0.02 U of RNase-free DNase I (Thermo Sci-entific, Waltham, MA, USA) was added and incubated at 37°C for 2 min to degrade unprotected DNA. Finally, 1/10 (v/v) of 50 mM EDTA was added to each reaction and in-cubated at 65°C for 10 min to stop the reaction. DNAs in the samples were extracted using GeneJET PCR Purifica-tion kit (Thermo Scientific, Waltham, MA, USA) and sent for fragment length analysis by capillary electrophoresis in Eurofins Genomics company (Ebersberg, Germany). Elec-tropherograms were aligned using GeneMapper v2.6.3 (Ap-plied Biosystems).

RESULTS

***S. islandicus* Δ orc1-2 showed hypersensitivity to NQO treat-ment**

First, *S. islandicus* Δ orc1-2 strain constructed previously (15) was investigated for its sensitivity to NQO, a drug that forms stable bulky quinolone adducts on bases of DNA in bacterial and eukaryotic cells, leading to the formation of DSBs (34,35), and it induces programmed cell death in *S. is-landicus* (16). Both Δ orc1-2 and its corresponding WT were grown in SCV media containing different concentrations of NQO. Growth of these cultures was monitored by measur-ing their A_{600} values. We found that 2.5 μ M NQO com-pletely inhibited the growth of the mutant while it required 4 μ M NQO to stop the growth of the WT strain (Supplemen-tary Figure S2), indicating that Δ orc1-2 is hypersensitive to this drug, in reference to WT.

The sensitivity of WT and Δ orc1-2 to NQO was also evaluated by determination of their survival rate after drug treatment. Both strains were again grown in SCV in the presence of different concentrations of the drug for 6 h (hours post treatment, hpt). The number of viable cells in all cultures was then estimated by determination of their colony formation units (CFU), with the results summarized in Table 1. Two features are evident in these data: (a) the number of viable cells in NQO-treated cultures exhibited a strong reverse correlation to the drug concentration for both Δ orc1-2 mutant and the WT strain, and (b) the ratio of cell viability between WT and Δ orc1-2 changed from 3.8 to 30 folds as the NQO content increased from 1 to 3 μ M

in the medium. These results confirmed the hypersensitivity of $\Delta orc1-2$ to NQO treatment.

Orc1-2 deficiency by gene deletion eliminated the NQO-responsive expression

Next, we extracted total RNAs from cell samples of WT and $\Delta orc1-2$ cultures (grown in SCV containing 2 μ M NQO) at 6 hpt and from the corresponding cell samples of untreated reference cultures. These RNA samples were employed for determination of mRNA abundance of individual genes by RNA-Seq. FPKM plot analysis of the RNA-Seq data revealed a large number of DEGs upon NQO treatment in WT, which did not show NQO-responsive expression in $\Delta orc1-2$ (Figure 1A, Supplementary Tables S3, S4 and S5). These include a total of 646 genes among which 290 are up regulated and 356 are down regulated (with a corrected P -value = 0.005, fold change > 2) (Supplementary Table S3, Figure 1B).

Many up-regulated DEGs could be implicated in DNA damage repair (Supplementary Table S4), including: (a) *orc1-2* coding for one of the three Orc1/Cdc6 orthologous proteins in this archaeon (20), (b) genes in the gene operon of UV-inducible pili of *Sulfolobus* (the Ups system) (36) and genes of the crenarchaeal system for exchange of DNA (the Ced system), the latter of which has recently been shown to be responsible for intercellular DNA transfer in *Sulfolobus* (37), (c) genes coding for the basal transcriptional factors TFB1 and TFB3 (38,39) and (d) genes coding for proteins involved in homologous recombination repair (40,41).

Several highly repressed DEGs are implicated in different cellular processes including replication initiation (Orc1-1 and Orc1-3) (42–45), genome maintenance and segregation (chromatin protein Sul7d and Cren7 (46) and chromosome segregation proteins SegA and SegB (47)), and cell division (CdvA, ESCRT-III and Vps4 (48,49) as well as several ESCRT-III paralogs (50), Supplementary Table S5). Together, these results suggested that the NQO-induced DNA damage could impose DNA replication inhibition and cell cycle arrest in *S. islandicus*.

RNA-Seq analysis also revealed that 37 genes showed differential expression in $\Delta orc1-2$ (9 up- and 28 down-regulated genes Figure 1C). Among the down regulated genes, 24 showed a <3 folds of repression whereas the remaining four genes had 3- to 5-fold reduction. The relatively low levels of repression suggest that the observed changes may reflect background fluctuation of gene expression in the mutant. The up regulated genes include six genes of 2- to 5-fold activation and three highly activated genes: SiRe_0629 and SiRe_0630 (9–10 folds), SiRe_0655 (47 folds), all of which are of unknown function. To this end, these data indicated that $\Delta orc1-2$ is no longer capable of mediating NQO-responsive expression.

***S. islandicus* $\Delta orc1-2$ lost the capability of cell aggregation and cell cycle regulation**

To further study the phenotype of the *S. islandicus* $\Delta orc1-2$, the deletion mutant and WT were grown in the medium containing 2 μ M NQO for 24 h. Cell samples were taken during incubation and examined for cell aggregates under

microscope. We found that WT formed small cell aggregates of 3–7 cells at 6 hpt, and larger cell aggregates (10–30 cells) appeared at 12 hpt (Figure 2A). In contrast, there was essentially no difference in cell aggregation between the NQO-treated $\Delta orc1-2$ cells and their corresponding untreated references since <5% of cells were found in aggregates and that number did not change after drug treatment (Figure 2B). Therefore, these results indicated that $\Delta orc1-2$ has lost the capability of cell aggregation.

These cell samples were also analyzed by flow cytometry. As shown in Figure 2C, the population of cells with DNA content clustering at 1 chromosome (G1+, comprising of G1 and ‘apparent G1’ cells, the latter of which contain 1 chromosome with fired origins of replication) slightly increased in WT at 6 hpt (<15%), and strikingly, G1+ cells accounted for 75% of the total cell population in the $\Delta orc1-2$ cell sample. These results suggested that cell division was inhibited in WT cells, but not in $\Delta orc1-2$ cells, consistent with the RNA-Seq data in which the expression of *cdvA*, *cdvB* and *vps4*, which code for the proteins (CdvA, ESCRT, Vsp4) responsible for the ESCRT mode of cell division in *Sulfolobus* (48,49), was down regulated in NQO-treated WT cells, but their expression was not changed in NQO-treated $\Delta orc1-2$ cells (Supplementary Table S5). In addition, the expression of *orc1-1*, *orc1-3*, which code for replication initiators responsible for initiation of *oriC1* and *oriC2* of the *S. islandicus* chromosome (15), was also inhibited in NQO-treated WT cells but not in NQO-treated $\Delta orc1-2$ cells (Supplementary Table S5), and these results suggested that replication initiation could have been inhibited in the WT cells but not in the $\Delta orc1-2$ cells. We reasoned that most G1+ cells could be ‘apparent’ G1 cells containing fired origins of replication on their chromosome but the DNA replication could be blocked by NQO-induced DNA lesions on the chromosome in the cells. If so, collapse of stalled replication forks would induce DSBs, leading to cell death. Indeed, continuous incubation of these NQO-treated cultures led to cell death to most $\Delta orc1-2$ cells as well as a fraction of NQO-treated WT cells (Figure 2C). To this end, our results indicated that the Orc1-2-dependent DDR regulation is of crucial importance to genome integrity maintenance in this archaeon.

Promoters of DDR genes mediated NQO-responsive expression

In bacteria and archaea, short DNA segments immediately upstream of genes often contain all promoter elements required for directing the expression of the genes. To test if promoters of DDR genes could also contain DNA motifs required for DDR regulation, promoter fragments of four DDR genes, *i.e.* *upsA*, *upsE*, *tfb3* and *cedA1*, were amplified by PCR using the primers list in Supplementary Table S2, giving a ca. 200-bp DNA fragment (upstream of the start codon) for each promoter. PCR fragments of these promoters were then used to replace the *araS*-SD promoter in pSeSD (27), yielding reporter gene plasmids for these DDR genes (listed in Supplementary Table S1). All these promoters were found to confer NQO-responsive expression from the reporter gene plasmids in *S. islandicus* (see below). Then, promoter sequences (100 bp preceding the

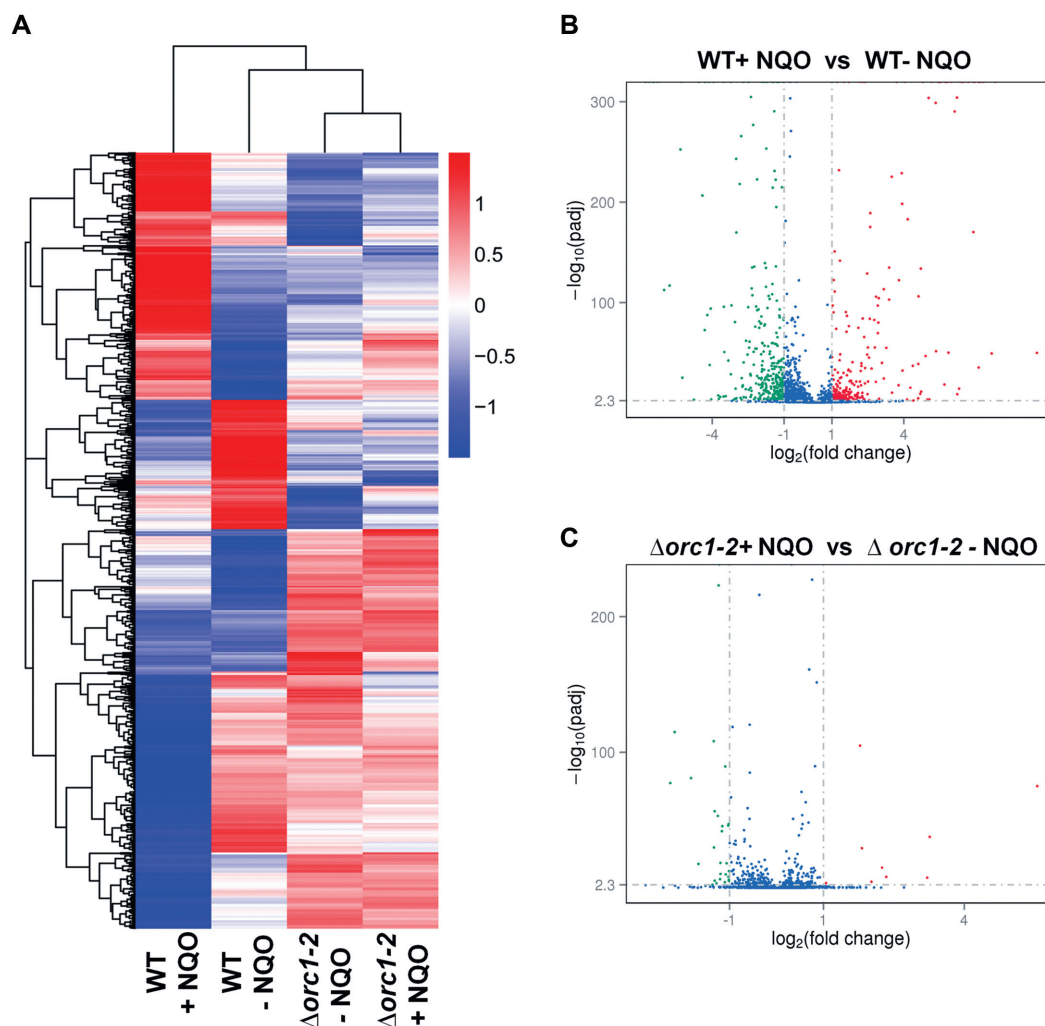


Figure 1. Global transcriptional change mediated by Orc1-2 upon NQO treatment. (A) Heatmap of the genome expression of *S. islandicus* E233S1 (WT) and Δ *orc1-2*. Both strains were grown in the presence or absence of 2 μ M NQO (indicated as + NQO and - NQO, respectively) for 6 h. Cell mass was collected from which total RNAs were prepared and used for RNA-Seq analysis. Genes are clustered with their log₁₀(FPKM+1) values and their expression levels are illustrated with different colors with red colors representing the highest levels of expression whereas blue ones indicating the lowest levels of expression. (B) Volcano plot of differentially expressed genes in the WT strain. (C) Volcano plot of differentially expressed genes in Δ *orc1-2*. X-axis: fold change in gene expression; Y-axis: statistical significance of the fold change. Genes exhibiting >2-fold (i.e. $-1 > \log_2 > +1$) up and down regulation are highlighted in red and green, respectively, whereas those that showed a <2-fold change in differential gene expression are shown in blue.

start codon) of 21 highly up regulated DDR genes (over 18 folds change, Supplementary Table S6) were retrieved from the genome sequence of *S. islandicus* REY15A (20) and analyzed for conserved sequence motifs using the MEME (Multiple EM for Motif Elicitation) suite (32). A 20 bp consensus (5'-AATAGTTTCRGWDTACTCWS-3') was identified, containing the DNA motif of 5'-ANTTTC-3' previously reported for UV-responsive gene promoters of *S. acidocaldarius* (51). The motif is positioned at -23 to -55 bp upstream of the ATG codon in most identified DDR genes (Supplementary Figure S3).

Next, the hexanucleotide motif (5'-ANTTTC-3') in four promoters (*upsA*, *upsE*, *tfb3* and *cedA1*) was mutated individually by transversion mutation, giving respective mutated promoters. Both the native promoters and their mutated derivatives were analyzed for NQO-responsive expression in WT and Δ *orc1-2*. In WT transformants, mutation

of the 5'-ANTTTC-3' motif completely abolished the NQO-responsive expression from each promoter (Figure 3), indicating the motif functions as an NQO-responsive element on these promoters. By contrast, none of the promoters showed the NQO-responsive expression in the Δ *orc1-2* mutant (Figure 3), suggesting that the 5'-ANTTTC-3' motif and the Orc1-2 protein could interact with each other to mediate DDR regulation in this archaeon.

Orc1-2 bound to the conserved motif on DDR gene promoters

To test if Orc1-2 could bind to the 5'-ANTTTC-3' motif present in the promoter regions of DDR genes, the *S. islandicus* *orc1-2* gene was cloned into pET30a, an *E. coli* expression vector, giving pET-*orc1-2*. The expression plasmid was introduced into *E. coli* for overexpression of Orc1-2 recombinant protein. Highly purified Orc1-2 recombinant protein was obtained (Supplementary Figure S4). DNase

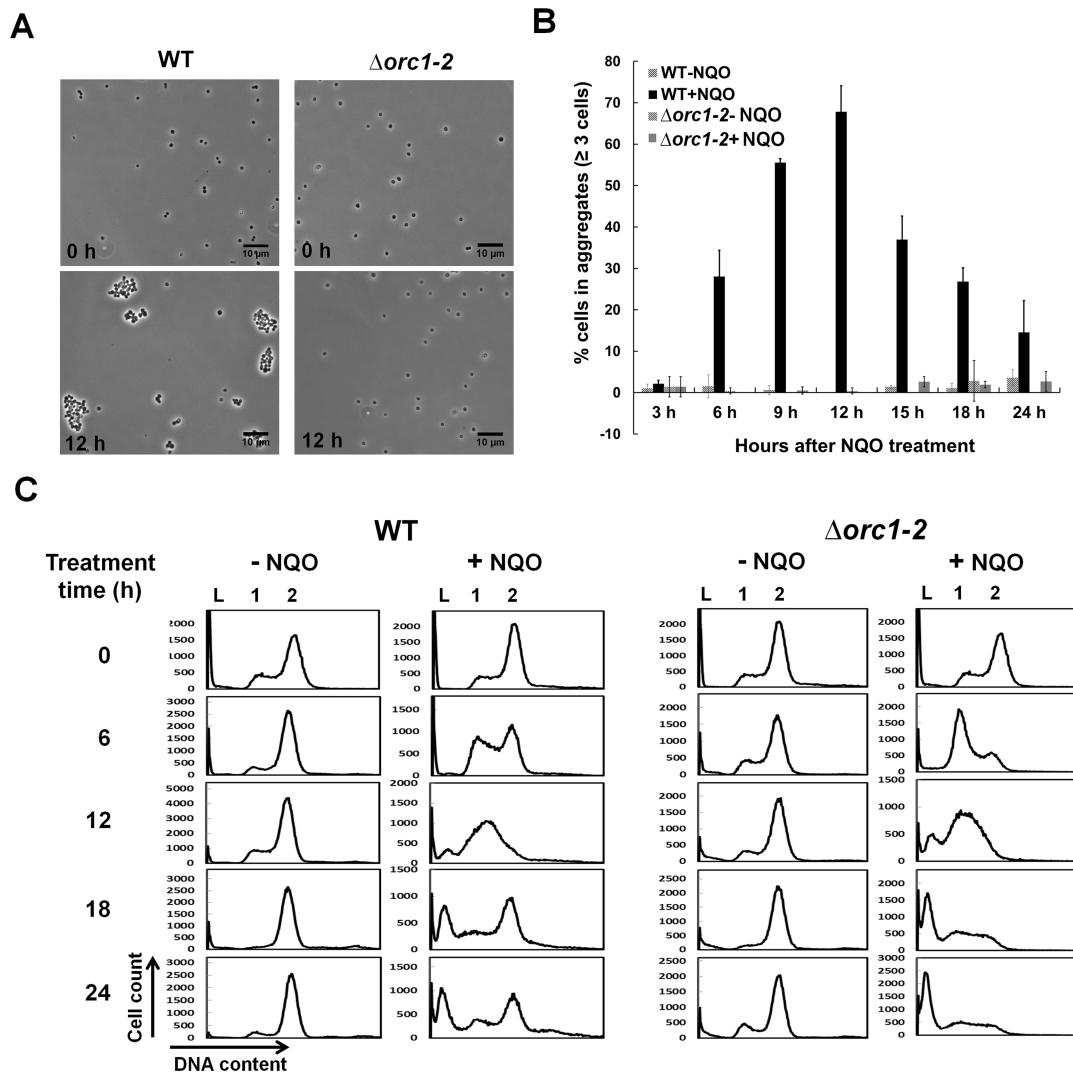


Figure 2. $\Delta orc1-2$ mutant lost the capability of cell aggregation and cell cycle regulation. (A) Formation of cell aggregates before (0 h) and after treatment with 2 μ M NQO (12 h). (B) Quantification of the extent of cell aggregation. (C) Cell cycle profiles of the cultures. DNA contents were divided into 256 arbitrary points on the X-axis, and cell counts (Y-axis) were obtained for each point and used to plot against the DNA content. WT: *S. islandicus* E233S1; $\Delta orc1-2$: *orc1-2* deletion mutant derived from the E233S1; DNA-less cells (L); cells containing one chromosome (1), and cells containing two chromosomes (2). Error bars: standard derivations of three independent experiments.

I footprinting assay was performed with the original and mutated promoters of *upsE* and *tfb3* genes, in the presence, or absence of Orc1-2 protein, following the procedure described previously (33). We found that Orc1-2 protein protected the sequence of 5'-ANTTTC-3' motif and its flanking regions on both DNA strands (Figure 4A), and substitution of the 5'-ANTTTC-3' motif on the mutated promoters completely abolished the protection of the DDRE region by Orc1-2 binding (Figure 4B). These results, together with the report genes assays shown in Figure 3, indicated that Orc1-2 protein binds specifically to 5'-ANTTTC-3' on these DDR gene promoters and activates their expression.

S. islandicus cells stably expressing a low level of Orc1-2 protein exhibited hypersensitivity to NQO treatment

To yield an insight into the DDR regulation by Orc1-2 in this archaeon, we constructed a *S. islandicus* strain in

which the original promoter of the *orc1-2* gene was replaced with *araS-50*, a promoter derivative of the *araS* gene coding for an arabinose-binding protein. This yielded the promoter-substitution mutant designated *orc1-2^{araS}* (Supplementary Figure S5). It has been shown that the *araS-50* promoter confers arabinose-inducible expression in this archaeon (30). Therefore, when cultured in SCV with sucrose as the carbon source, a non-inducible medium for the *orc1-2^{araS}* gene, Orc1-2 should be expressed to a constantly low level in the mutant. Then, WT and *orc1-2^{araS}* were grown in SCV for 48 h either in the presence or absence of 2 μ M NQO. Cell samples were taken during incubation and examined for *orc1-2* expression, culture growth and cellular DNA content.

Analysis of Orc1-2 protein in the WT and *orc1-2^{araS}* cells by immunoblotting revealed that the cellular content of Orc1-2 remained constantly low (Figure 5A) in the presence

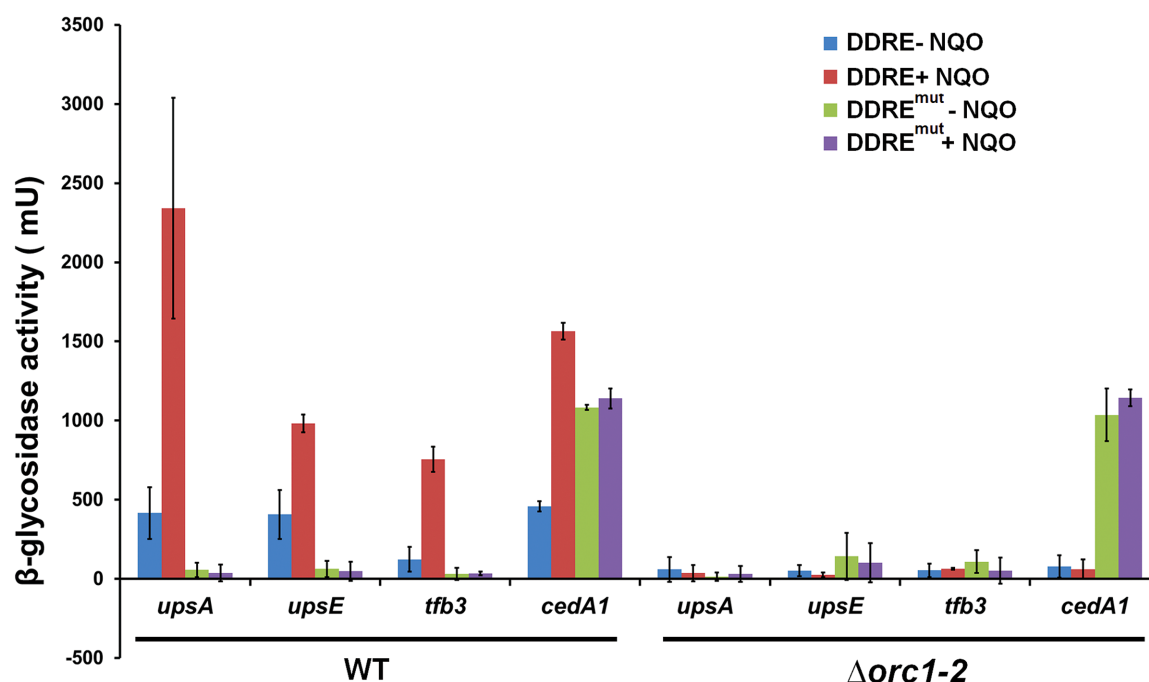


Figure 3. Promoters of NQO-inducible genes dictate the regulation of the NQO-responsive expression. Four highly up regulated genes (*upsA*, *upsE*, *tfb3* and *cedA1*) were chosen for the reporter gene assay. *S. islandicus* strains carrying one of the 8 reporter gene plasmids were grown in SCV in the presence, or absence of 2 μ M NQO (denoted as +NQO and -NQO respectively) for 6 h, and cell mass was collected from which cell extracts were prepared and used for determination of β -glycosidase activity. DDRE: reporter gene plasmids of original promoters containing the 5'-ANTTTC-3' motif; DDRE^{mut}: reporter gene plasmids of mutated promoters carrying transversion mutation in the 5'-ANTTTC-3' motif; WT: the genetic host *S. islandicus* E233S1; Δ *orc1-2*: *S. islandicus* *orc1-2* deletion mutant. Error bars: standard derivations of three independent experiments.

of NQO, consistent with the nature of the *orc1-2*^{araS} fusion gene. The mutant showed an interesting phenotype: it grew in a similar fashion as for WT in the absence of NQO; however, mutant growth was strongly inhibited in the presence of the drug and the growth inhibition persisted (Figure 5B). These results suggest that *orc1-2*^{araS} could be deficient in initiating DDR regulation as shown for Δ *orc1-2* (compare with the data in Figure 2 and Supplementary Figure S2). Indeed, flow cytometry of the cell samples showed that, a large number of *orc1-2*^{araS} cells became DNA-less cells during incubation in the NQO-SCV medium, and this is in contrast to the WT cells grown in the same medium in which majority of cells were recovered from NQO treatment at 48 hpt (Figure 5C). Together, these results indicated that *S. islandicus* cells containing a low level of Orc1-2 protein fail to respond to NQO treatment and they are as hypersensitive to NQO treatment as for Δ *orc1-2* cells.

S. islandicus cells stably expressing a high level of Orc1-2 protein responded more promptly to NQO treatment

Next, we investigated how the archaeal cells containing a high level of Orc1-2 could respond to NQO treatment. To do that, both *orc1-2*^{araS} and WT were grown in ACV with D-arabinose as the carbon source, in the presence or absence of 2 μ M NQO. This medium is an inducible medium for the expression of the *orc1-2*^{araS} gene such that Orc1-2 should be expressed to a constantly high level in *orc1-2*^{araS} cells. These cultures were grown for 48 h during which cell samples were taken for the analyses as above described. First,

immunoblotting confirmed that Orc1-2 was expressed to a high level in ACV-cultured *orc1-2*^{araS} cells (Figure 6A). Then, growth data revealed that NQO treatment had little influence on the growth of *orc1-2*^{araS} cells in ACV since very similar growth curves were obtained for NQO-treated versus untreated *orc1-2*^{araS} cultures (Figure 6B). Nevertheless, flow cytometry detected the increase of the cell population of 1–2 chromosomes in the NQO-treated cultures of both strains, indicating that both WT and *orc1-2*^{araS} are capable of recovering from NQO-induced DNA damage under this growth condition (Figure 6C). Strikingly, both sets of data (growth curves and flow cytometry profiles) suggest a quick recovery for the promoter substitution mutant, relative to WT (Figure 6). Taken together, these results suggested that a high level of Orc1-2 could probably shorten the time required for execution of DDR regulation in *S. islandicus*.

A constant high level of Orc1-2 protein enabled immediate induction of DDR genes in the archaeon upon NQO treatment

To test that, the activation of gene expression in *orc1-2*^{araS} and WT cells after NQO treatment was investigated for a few selected DDR genes, including *tfb3*, *upsX* and *cedB*. Cell samples taken at the early stage of NQO treatment (1 and 3 hpt) were used for total RNA extraction, and the extracted RNAs were analyzed for DDR gene expression by RT-qPCR.

As shown in Figure 7A, none of the three DDR genes showed any NQO-responsive expression in the cells expressing a constant low level of Orc1-2 (SCV-cultured *orc1-2*^{araS}

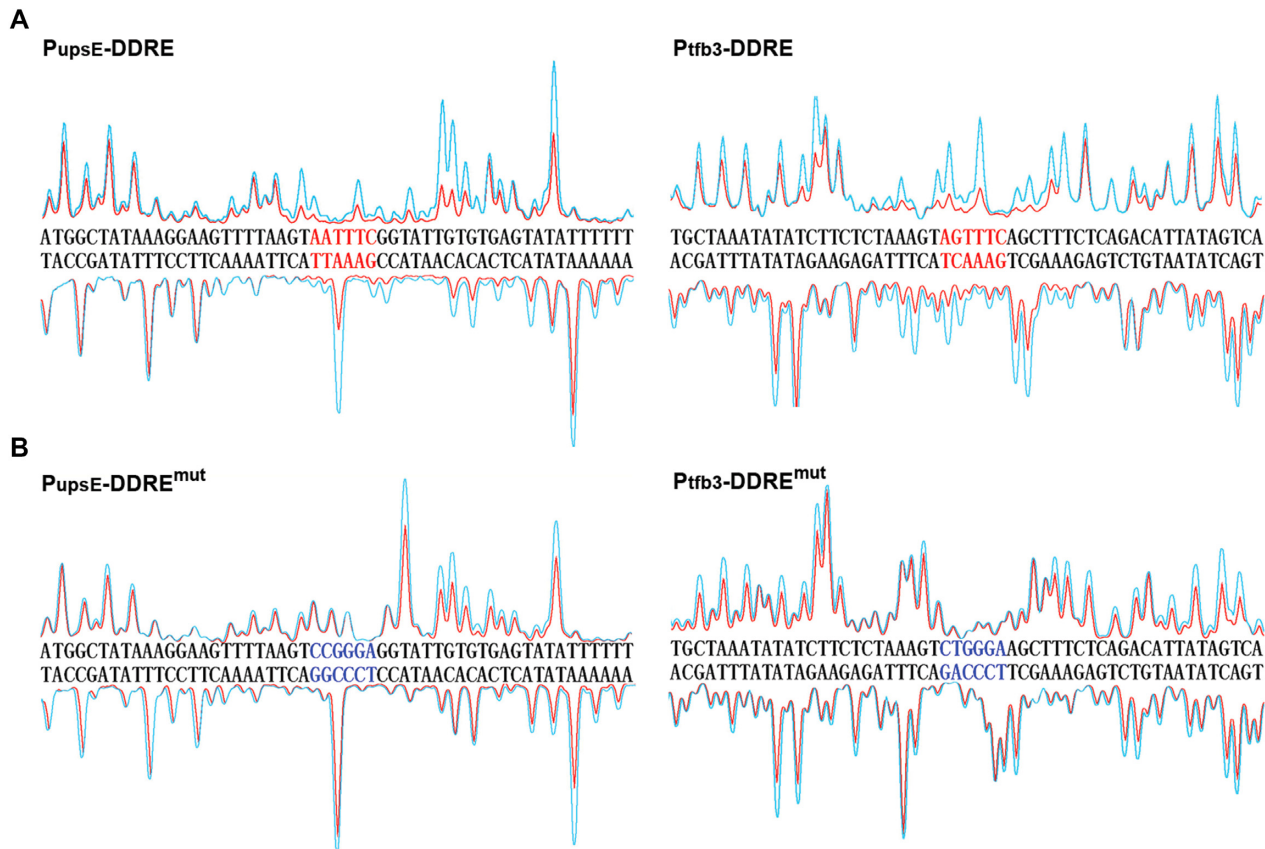


Figure 4. Orc1-2 protein bound to a conserved motif on *upsE* and *tfb3* promoters. DNase I footprinting was performed with fluorescence-labeled DNA fragment of *P_{upsE}* or *P_{tfb3}* promoter (ca. 200 bp) in the presence (red peaks) or absence (cyan peaks) of Orc1-2. DDRE: Promoter fragments containing the 5-ANTTTC-3' motif (highlighted in red); DDRE^{mut}: mutated promoter fragments carrying the transversion mutation of the 5-ANTTTC-3' motif (highlighted in blue). The promoter sequences shown for *P_{upsE}* and *P_{tfb3}* are positioned from -17 to -69 and from -47 to -99 in reference to their start codons, respectively.

cells), reminiscent of the lack of the DDR regulation in Δ *orc1-2*. By contrast, all three DDR genes were readily activated by NQO treatment in the cells expressing a constantly high level of the regulator (ACV-cultured *orc1-2^{araS}* cells), and in fact, their expression levels in *orc1-2^{araS}* cells at 1 hpt are higher than those in WT cells at 3 hpt (Figure 7B). In summary, two sequential events have been identified in the archaeal DDR regulation: (a) activation of the expression of Orc1-2, and (b) the subsequent activation of target genes by Orc1-2. Therefore, Orc1-2 plays a very important role in the DDR regulation in this archaeon.

DISCUSSION

Here, we show that Orc1-2 functions as a global regulator to mediate DNA damage response in *S. islandicus*, a hyperthermophilic crenarchaeon. To our knowledge, this represents the first identification of a key regulator in DNA damage response of an archaeal organism.

S. islandicus Orc1-2 is an ortholog of the archaeal/eukaryotic Orc1/Cdc6 superfamily of replication initiators (42–45). The encoding gene is not closely located to any origins of replication on the chromosome of *S. islandicus* (15), and this genetic organization is conserved in all known species in Sulfolobales (44,45) (Supplementary

Figure S6). A previous genetic analysis of *orc1-2* function in *S. islandicus* revealed that the gene does not have a function in replication initiation, in contrast to its orthologs Orc1-1 and Orc1-3 that function as the replication initiator to the adjacent *oriC1* and *oriC2* individually (15). Here, we show that Δ *orc1-2* is hypersensitive to NQO, a chemical that yields bulky adducts on bases of chromosomal DNAs, which are to be repaired by NER (35), and the mutant has lost the capability to form NQO-induced cell aggregation and cell cycle regulation (Figure 2). In fact, NQO-responsive expression is completely abrogated in Δ *orc1-2* (Figure 1, Supplementary Table S3). Together, these findings suggest that, during evolution, Orc1-2 has lost the function as a replication initiator and gained the new function as a primary regulator in DNA damage response. In addition, investigation of bacterial replication initiator DnaA proteins and eukaryotic replication initiators Orc1 and Cdc6 proteins reveals that these factors also function as transcriptional regulator, but these factors have maintained their function in replication initiation (52–54), which is in contrast to the scenario reported for the *S. islandicus* Orc1-2 here. Therefore, this Orc1-2 protein represents the first example of functional diversification of proteins of the Orc1/Cdc6 superfamily.

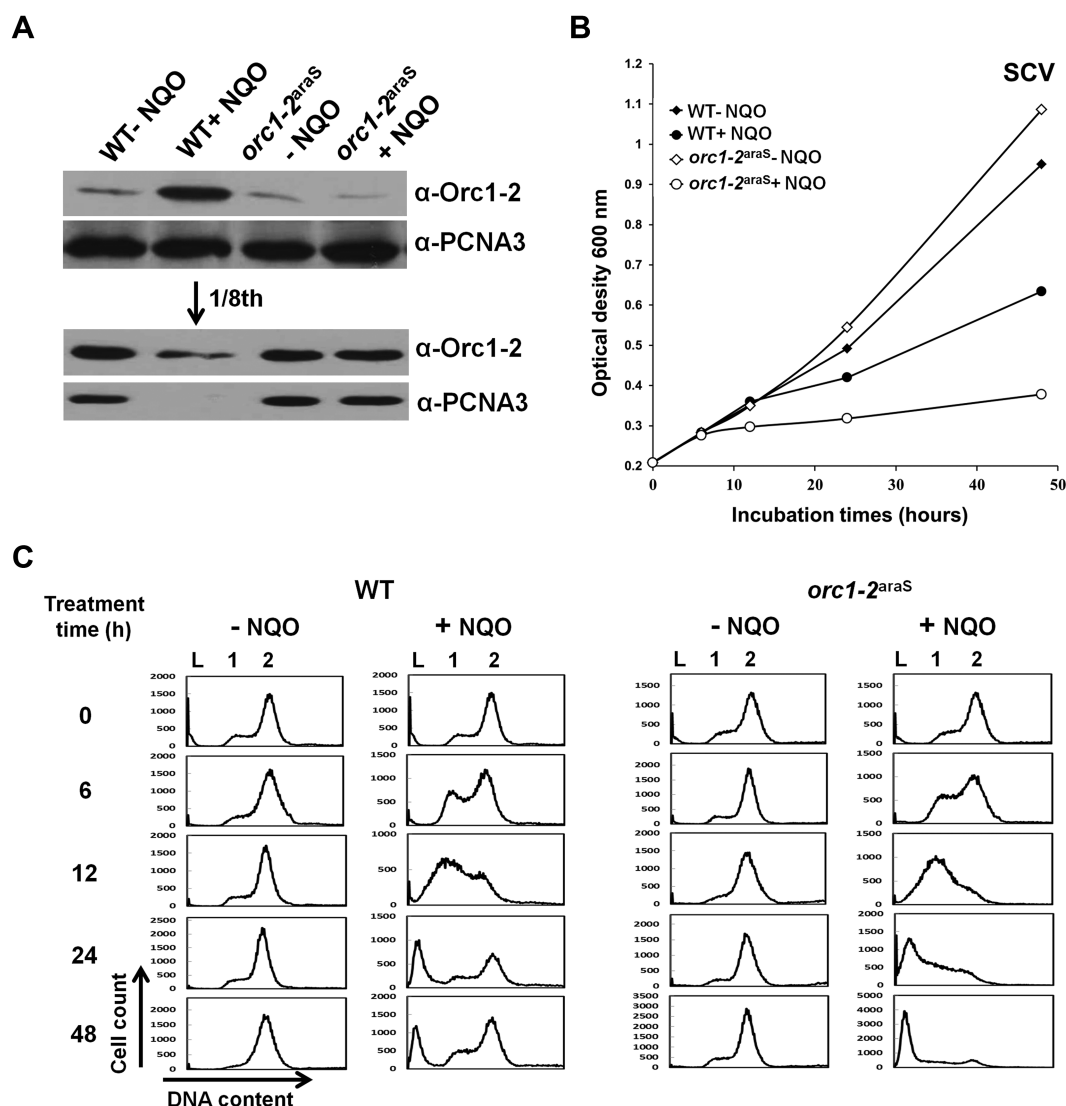


Figure 5. *S. islandicus* Orc1-2^{araS} cells failed to respond to NQO treatment in SCV media. (A) Western analysis of Orc1-2 protein. Only the samples taken at 6 hpt were shown. In the lower panel, only NQO-treated WT sample (lane 2) was diluted for 8 folds. PCNA3 (one of the subunits of the replication clamp) was used as the loading reference. (B) Growth curves based on absorbance at 600 nm. (C) Flow cytometry profile of cell samples. DNA contents were divided into 256 arbitrary points on the X-axis, and cell counts (Y-axis) were obtained for each point and used to plot against the DNA content. WT: *S. islandicus* E233S1; *orc1-2^{araS}*: Promoter-substitution mutant containing the *araS*-50 promoter-*orc1-2* fusion gene. DNA-less cells (L); cells containing one chromosome (1), and cells containing two chromosomes (2).

Interestingly, it has been reported that archaeal Orc1/Cdc6 proteins form two distinct clades corresponding to Orc1-1 and Orc1-2 clusters in which proteins of the Orc1-2 cluster evolve faster than those of the Orc1-1 cluster (44,45). Since *S. islandicus* Orc1-2 functions as a global DDR regulator, the fast evolution of the Orc1-2 cluster probably reflects the acquisition of a new function for some of Orc1 proteins in this cluster. Furthermore, the conservation of the genetic organization of *orc1-2* and its flanking genes in Sulfolobales (Supplementary Figure S6) suggests that these Orc1-2 homologs could also function as a key regulator in archaeal DDR regulation. Moreover, it is also very tempting to investigate whether non-Sulfolobales members of the Orc1-2 cluster could also play a role in

DNA damage-induced genome expression as for the *S. islandicus* Orc1-2.

Our research has gained important insights into the mechanisms of Orc1-2-dependent regulation in *S. islandicus*. First, a conserved motif (5'-ANTTTC-3') is present in the promoters of a number of highly up regulated DDR genes, and it is identical to the motif identified in UV-inducible genes of *S. acidocaldarius* (51). Here, we show that the *S. islandicus* Orc1-2 protein binds specifically to the motif present in a selected set of DDR gene promoters *in vitro* (Figure 4), and inactivation of either component (Orc1-2 deficiency or mutagenesis of the DNA binding motif) abolishes the NQO-responsive expression from all tested promoters in reporter gene assay (Figure 3). To this end, the deduced mechanism of transcriptional activa-

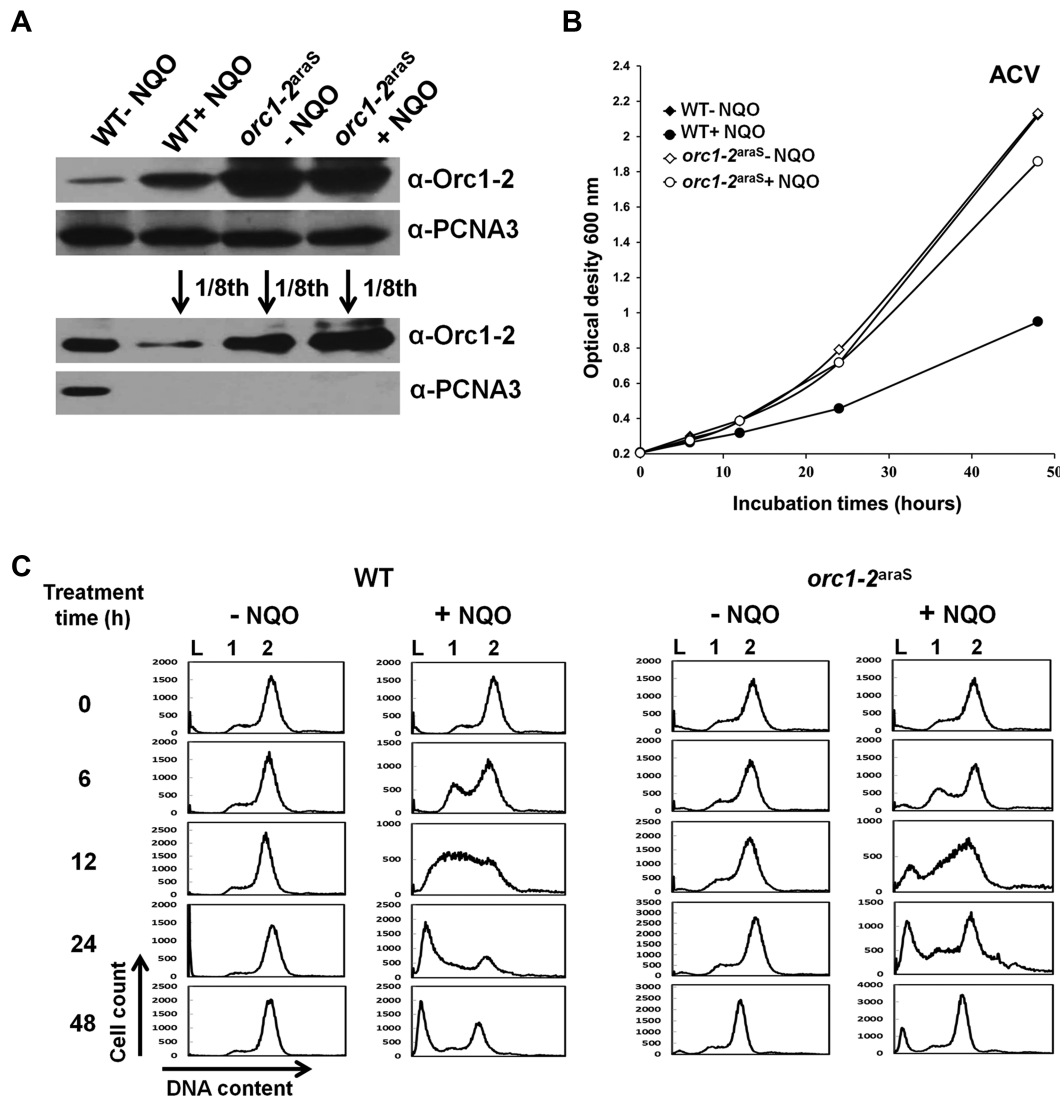


Figure 6. *S. islandicus* Orc1-2^{araS} cells exhibited a quicker recovery from NQO treatment in ACV media. (A) Western analysis of Orc1-2 protein. Only the samples taken at 6 hpt were shown. In the lower panel NQO-treated WT sample (lane 2), NQO-treated and untreated *orc1-2^{araS}* samples (lane 3 and 4) were diluted for 8 folds. PCNA3 (one of the subunits of the replication clamp) was used as the loading reference. (B) Growth curves based on absorbance at 600 nm. (C) Flow cytometry profile of cell samples. DNA contents were divided into 256 arbitrary points on the X-axis, and cell counts (Y-axis) were obtained for each point and used to plot against the DNA content. WT: *S. islandicus* E233S1; *orc1-2^{araS}*: Promoter-substitution mutant containing the *araS*-50 promoter-*orc1-2* fusion gene. DNA-less cells (L); cells containing one chromosome (1), and cells containing two chromosomes (2).

tion is that Orc1-2 binds to the DDR promoters and facilitates gene expression. Second, a high level of *orc1-2* expression is essential but not sufficient to trigger DDR regulation in this archaeon, and this is strongly supported by the following findings: (a) When *orc1-2* is constitutively expressed to a constantly low level in *orc1-2^{araS}*, a promoter-substitution mutant, DDR is not induced by NQO treatment as observed for Δ *orc1-2*. (b) When *orc1-2* is expressed to a constantly high level, the mutant does not show any growth delay or retardation in the absence of NQO. Third, *orc1-2^{araS}* cells grown in ACV media contain a high level of Orc1-2 but they do not exhibit any DDR regulation in the absence of NQO. Nevertheless, these *orc1-2^{araS}* cells respond more promptly to NQO treatment in DDR initiation than WT cells (Figure 7). In addition, TFB3 is a truncated TFB paralog that also functions as a DDR regulator to a

subset of DDR genes (31). However, the TFB3-dependent activation of DDR genes is completely abolished in Δ *orc1-2*, indicating that TFB3 must function downstream of Orc1-2 in the DDR network of this archaeon (Supplementary Table S4). Taken together, these results suggest that Orc1-2 is the primary regulator in the DDR network in this archaeon and that the factor could be activated by posttranslational modifications (PTMs), in analogy to the eukaryotic ATM-dependent activation of DDR regulators, such as p53, an extensively characterized tumor suppressor that regulates cell cycle arrest, DNA repair and apoptosis (55,56).

Genes that are regulated both by Orc1-2 and by TFB3 include those present in the *ups* operon coding for the proteins involved in the UV-responsive pilus formation (36) and those of the Ced system that mediates intercellular DNA transfer (37). It has been known for a long time that *S.*

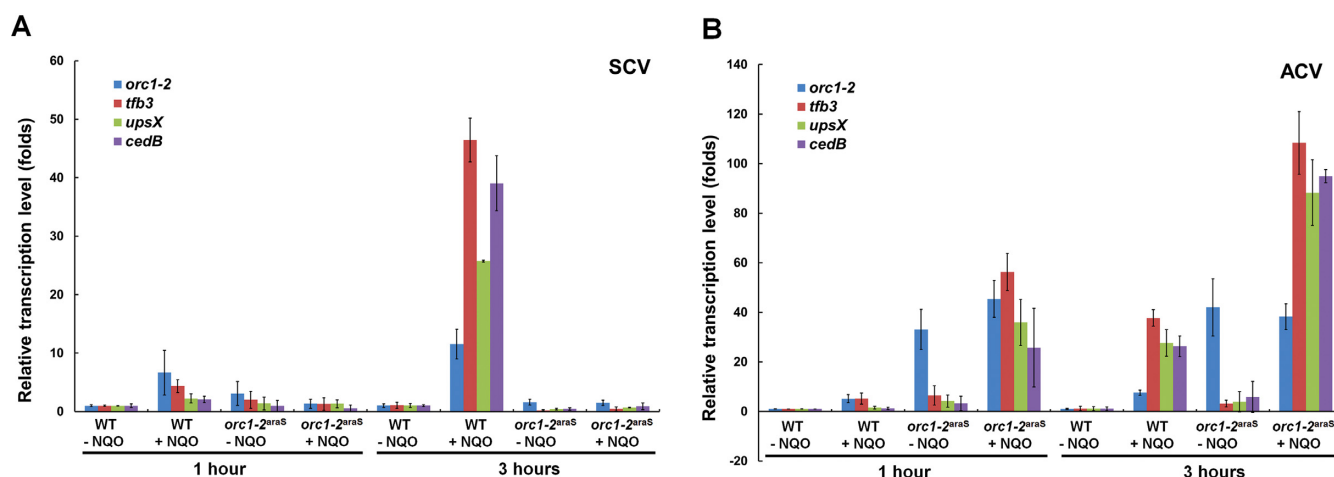


Figure 7. High Orc1-2 level in *S. islandicus* cells prior to NQO treatment enabled earlier responsive expression. (A) Relative expression levels of *tfb3*, *upsX* and *cedB* in WT and *orc1-2^{araS}* cells at 1 and 3 hpt when grown in SCV in which the expression level of *orc1-2* was constantly low. (B) Relative expression levels of *tfb3*, *upsX* and *cedB* in WT and *orc1-2^{araS}* cells at 1 and 3 hpt when grown in ACV in which the expression level of *orc1-2* was constantly high. The expression level of these genes in the cell samples of WT and *orc1-2^{araS}* was estimated by RT-qPCR with the obtained data normalized to the level of 16S RNA. WT: *S. islandicus* E233S1; *orc1-2^{araS}*: Promoter-substitution mutant containing the *araS-50* promoter-*orc1-2* fusion gene. Error bars: standard derivations of three independent experiments.

acidocaldarius efficiently mediates chromosomal DNA exchange (57) and the process is highly efficient (58). In fact, other *Sulfolobus* species have also been shown to mediate DNA conjugation although the process has to be induced such as by UV irradiation or DNA damage treatment with bleomycin (36,37,59). Since the two systems have been implicated in repairing UV-induced DNA damage in *S. acidocaldarius* (36,37,59,60) and NQO-mediated DNA damage in *S. islandicus*, activation of their gene expression represent one of the DDR network of cellular events in *S. islandicus*.

The up regulation of known HRR genes including *nurA*, *rad50*, *mre11* and *herA* (Supplementary Table S4) by DNA damage agents is consistent with the requirement for DNA repair with high fidelity and the strong activation of DNA transfer activity for importing DNA template for HRR as discussed above. Currently, whether there is any functional connection between the DNA transfer and HRR activity remains to be investigated. Employment of the existing genetic manipulation methods to tackle this problem is challenging since each HRR gene is essential in this organism (61,62). Nevertheless, an efficient CRISPR-based gene knockdown approach has been reported for this crenarchaeon recently (63) and successfully used to dissect the function of the essential *topR1* gene coding for a reverse gyrase (24). This approach should be useful to test the hypothesis that the HRR system works in concert with the Ced system in archaeal DNA damage repair.

Among the genes coding for the four DNA polymerases in *S. islandicus* REY15A (20), only SiRe_0614 and SiRe_0615 that code for DNA polymerase B2 (Dpo2) show NQO-responsive activation, and the regulation is Orc1-2-dependent but TFB3-independent, as demonstrated here and in a previous work. Strikingly, the *Sulfolobus* Dpo2 has been considered as an inactivated DNA polymerase because there are multiple substitutions in the catalytic residues of its polymerase and exonuclease domains (64). Nevertheless,

a recombinant protein of the large subunit of the *S. solfataricus* Dpo2 is capable of catalyzing DNA synthesis using DNA templates containing a range of DNA lesions, such as 8-oxoguanine, hypoxanthine and uracil (65), suggesting that it could be a translesion DNA polymerase. Since SiRe_0236 coding for the Y-family DNA polymerase (whose homolog in *S. solfataricus* functions as DNA lesion-bypass enzyme) did not exhibit any up regulation upon NQO treatment, it would be interesting to investigate if Dpo2 could be an active DNA polymerase in DNA damage repair.

In conclusion, our research has yielded the first picture of the archaeal DDR network for the regulation of cellular processes upon DNA damage, in which the key regulator, Orc1-2 is positioned at the heart of the regulatory network (Figure 8). First, the global regulator has to be activated by DNA damage, and the activation occurs in two aspects: (a) Orc1-2 strongly up regulates its own gene expression, and (b) the factor could be activated by PTMs, such as phosphorylation, acetylation and/or methylation. Then, the activated Orc1-2 exerts either activation or repression to the expression of a large number of DDR genes. The repressed genes include those that mediate cell cycle arrest, including inhibition of cell division, DNA replication initiation and genome segregation. The activated genes are as following: (a) Dpo2 that may function in translesion DNA synthesis, (b) HRR genes that function in DNA repair and (c) TFB3, a secondary DDR regulator responsible for activation of the expression of the *ups* operon, the *ced* genes (31), both of which have been implicated in importing DNA fragments for HRR. Finally, (d) the cell aggregation and DNA transfer systems are subjected to dual control by TFB3 and Orc1-2 (Figure 8). These results demonstrate, for the first time, that the strategy of orchestrating a network of cellular events to deal with DNA damage is evolutionarily conserved across the three domains of life.

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